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In Antibody-Directed Enzyme-Prodrug Therapy (ADEPT), antibody - enzyme constructs localize to tumor tissue the toxification of non-toxic prodrugs. Recombinant fusion proteins may overcome limitations of chemical conjugates regarding stability and size.

As a general model system, we have constructed fusion proteins of an antibody against the tumor antigen A33 with cytosine deaminase (CD), which converts 5-fluorocytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU). Using a T7 polymerase-based expression system, a plasmid vector was designed to allow cloning of the scFv either preceding or following the enzyme.

The fusion proteins were produced in *E. coli* and purified and renatured from inclusion bodies. Antibody and enzyme activities were confirmed by separate functional assays. To test the complete ADEPT system *in vitro*, A33-positive cell cultures were incubated with the fusion protein, washed, and cultured for 48h in the presence of 5-FC. While fusion protein or up to 1 mM 5-FC alone had no effect on cell growth, their sequential combination quantitatively increased median 5-FC toxicity. Preincubation with anti-A33 blocked this effect. A control fusion protein with GFP instead of CD had no effect on 5-FC toxicity.

While avidity of the constructs remains to be improved, these data prove the feasibility of scFv-based ADEPT in principle. Currently, this system is transferred using breast-cancer-specific antibodies F19 and 3S193.

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Table of Contents

Front Cover 1
Standard Form (SF) 298, Report Documentation Page 2
Foreword 3
Table of Contents 4
Introduction 5
Report Body 5
Key Research Accomplishments 10
Reportable Outcomes 10

Prodrug Therapy for Breast Cancer Targeted by Single-Chain Antibodies F19 and 3S193

Introduction

Antibody-directed enzyme-prodrug therapy (ADEPT) employs antibody-enzyme constructs to achieve tumor targeted activation of nontoxic prodrugs. Objective of this project is the engineering of recombinant fusion proteins of single-chain antibody fragments (scFv) and prodrug-activating enzymes. Its scope comprises the detailed investigation of this ADEPT approach with a variety of scFv-enzyme fusion proteins based on the antibodies F19 and 3S193 in vitro and in vivo, so as to eventually allow their evaluation with regard to clinical studies. The first 15 months were assigned to establishing the technology of cloning and expressing the protein constructs. Overcoming unexpected obstacles to the production of functional fusion proteins, the general methodology for construction and expression of such constructs has been developed. While during the report report this was only completed for one exemplary scFv and remains to be done with the antibodies named above, in having developed the complete methodology rather than the production of scFv alone the project is ahead of its schedule.

Report Body

The report period comprises Task 1 of the approved Statement of Work, the expression of recombinant single-chain antibodies F19 and 3S193 (months 1 – 14).

This task was formulated based on our existing experience at the time of the proposal with single-chain antibodies and fusion proteins of the huA33 anti-colon cancer antibody. As it turned out since, while small-scale production has been reproducible, considerable obstacles exist to the large-scale production of such fusion proteins, namely their toxicity to the host organism and their tendency to form insoluble aggregates when produced in higher concentrations. Therefore, work on A33-based fusion constructs had to

continue as part of this project in order to develop a general solution for this technical problem.

Isolation and cloning of variable region cDNA by RT-PCR

Plasmids for the expression of F19 single-chain antibody have been constructed as described in the grant proposal. The cDNA for the variable regions of this antibody had been isolated from the F19 hybridoma, and after insertion of suitable restriction sites by PCR the variable fragments were cloned into the existing pHOG-21 plasmid backbone (1). In the process of expression optimization described below, the completely assembled cDNA has then been excised and inserted into another bacterial expression vector, pET 25b(+). Both vector backbones carry 6 x histidine tags.

All cDNA sequences were verified by automated fluorescence sequencing and found to be correct. Protein expression was induced under standard conditions, and the proteins were isolated from the periplasmic space or from inclusion bodies and then purified by immobilized metal affinity chromatography (Talon, Clontech). The binding of the scFv was demonstrated by plasmon surface resonance and flow cytometry studies, confirming the expected specificity.

To avoid duplication of work, our present plan is to start the 3S193 scFv construct once complete in-vitro–testing of the existing constructs has confirmed that the current construction and expression methodology for fusion constructs can be employed on a routine basis.

Construction of antibody-enzyme fusion proteins

As mentioned above, the expression of complete fusion proteins was optimized on a proof-of-principle basis using the A33 scFv that we had previously generated.

Based on the scFv expression vectors described above, expression vectors for fusion proteins of scFv with the enzymes cytosine deaminase and thymidine kinase were constructed, and their identity and accuracy was confirmed by DNA sequencing. The detailed cloning strategy differed from that outlined in the proposal in that the enzyme DNAs were cloned into the existing scFv expression vectors. This procedure was chosen after it turned out that the scFv portion was more problematic to express in an active form

than the enzymes, and hence proper expression of the scFv gained priority in the optimization process.

For the same reason, we designed fusion constructs with the reverse order of the component parts, i.e. enzyme–antibody instead of antibody–enzyme, in order to test if the order of component fragments influences expression yield and function. These constructs, however, have not yet been functionally tested.

Production of scFv and fusion proteins in different expression systems

With the two expression vectors we tested two fundamentally different bacterial expression strategies. The pHOG-21 vector had been designed and optimized for periplasmic expression of soluble, functional scFv (1), whereas the strength of the pET vector system (Novagen, Madison, WI) lies in the high-yield production of proteins that are difficult to produce otherwise and, due to lack of mammalian chaperones and the high expression levels, tend to accumulate as inclusion bodies.

With the pHOG system, we were able to produce small amounts of the scFv's and fusion proteins, demonstrating the feasibility of our cloning strategy. The yield was too low, however, to produce enough material for in vivo studies or even for conclusive in vitro experiments.

The pET system is based on the mutual specificity of T7-RNA polymerase and the T7-promotor, leading to tight transcriptional control: During the growth face, the foreign T7-promotor is not recognized by the bacterial RNA polymerases, whereas, once its own expression is induced under an IPTG-inducible lac-Z promotor, T7-polymerase is produced in large amounts and transcribes T7 promotor-controlled genes with high efficacy.

In our experiments, the T7-system lead to high-level expression of scFv and fusion proteins. Both however, were almost exclusively retrieved as inclusion bodies requiring a separate refolding prodedure. Several refolding strategies have been tested by now, including mild resolubilization based on detergents such as lauroylsarcosine and subsequent dialysis against a Tris buffer, long-term refolding of highly diluted resolubilized protein in the presence of a redox pair and L-arginine, and matrix-bound refolding by gradual removal of the solubilizing agent.

While some of these experiments are still underway, this process has already been shown to increase the yield of functional protein. With the most basic method, detergent solubilization and dialysis, we were able to demonstrate the dual specificity of antibody binding and enzyme reaction in the A33–cytosine deaminase constructs (fig. 1). When antigen-positive tumor cells were cultured in the presence of the cytosine-deaminase fusion protein, washed, and then incubated with the prodrug, a considerable reduction in cell survival was observed (2). This effect could be blocked by pre-incubation with the native antibody, but not by preincubation with an isotypic control antibody. Neither the fusion construct alone nor the prodrug alone had any effect on cell survival at the concentrations used.

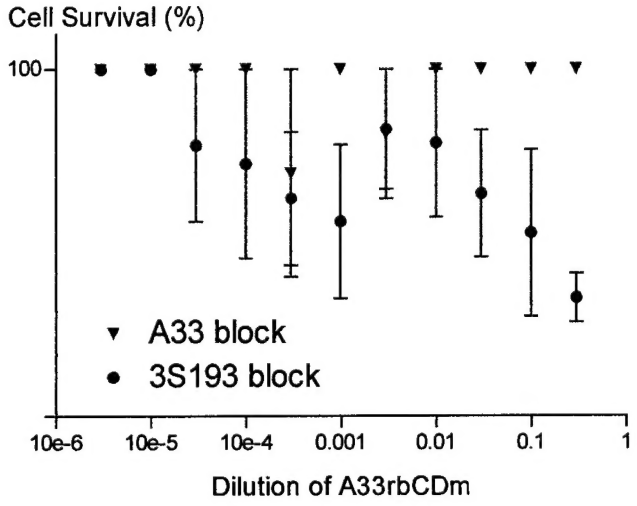

Fig. 1: A33-CD ADEPT system in vitro: A33-antigen-positive cells were incubated with the A33-CD fusion protein for two hours, washed three times, and then incubated with 5-FC. Percent cell survival after pre-saturation with A33 or with 3S193, which has here been used as an isotypic control antibody.

Despite the successful recovery of bifunctional protein, so far the results also show that the fusion proteins' affinity for antigen is lower than theoretically expected, whereas the enzyme activity was found to be similar to that of recombinantly expressed enzyme without the scFv. This observation may eventually be explained by the inherently lower avidity of the monovalent scFv compared to a bivalent IgG antibody or $F(ab)_2$ fragment. Should this observation hold true for the remaining antibody constructs, it may therefore

be necessary to modify this ADEPT concept so as to employ bivalent antibody constructs.

Educational aims and Summary

As expected in the grant proposal, the completed grant period has provided the principal investigator with improved skills in the empoyed methodologies in molecular biology and protein chemistry as well as deeper insight into the underlying molecular and immunological mechanisms.

The expression of heterobifunctional fusion proteins, while conceived and realized in principle when the grant proposal was submitted, posed technical challenges in achieving sufficiently high production yields for subsequent experiments. To solve these problems, the expression system was changed, and high yield production was achieved with the T7 polymerase expression system. While the majority of the produced protein is insoluble, sufficient functional protein has been generated for in vitro assays, demonstrating the feasibility of the selected approach. With this cloning and expression methodology in place, the project is well set to meet the specific aims for the second year of the grant period.

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Appendix

Key Research Accomplishments

- Cloning of protoypical scFv-enzyme fusion proteins and development of a general cloning and expression platform for the remaining constructs
- Development and optimization of an expression and refolding system for the production of functional bifunctional constructs
- Proof-of-principle demonstration of the scFv-based ADEPT system in vitro
- Construction of F19 single-chain antibody

Reportable Outcomes

Abstract and presentation:
Deckert, P. M., Ch. Renner, E. Richards, C. Williams Jr., J. R. Bertino, S. Welt, L. J. Old. Cloning and Expression of Recombinant Fusion Proteins of A33-Single Chain Antibodies and Cytosine Deaminase for Antibody-Directed Enzyme Prodrug Therapy. Proc. Amer. Assoc. Cancer Res. 41, abstract no. 1815 (2000)

Cloning and Expression of Recombinant Fusion Proteins of A33-Single Chain Antibodies and Cytosine Deaminase for Antibody-Directed Enzyme Prodrug Therapy

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Antibody-Directed Enzyme-Prodrug Therapy (ADEPT) is a tumor-targeting strategy that utilizes antibody-enzyme conjugates to localize to tumor tissue the conversion of non-toxic prodrugs into active chemotherapeutic agents. Recombinant fusion proteins of single-chain antibody fragments (scFv) and prodrug-converting enzymes offer a promising strategy to overcome the limitations of chemically linked conjugates, such as low stability and large diameter.

We have explored this strategy using single-chain fragments of the anti-colon carcinoma antibody huA33 and the enzyme cytosine deaminase (CD), which converts non-toxic 5-fluorocytosine (5-FC) into the TS-inhibitor 5-fluorouracil (5-FU). Based on the pHOG-21 vector, an expression cassette was designed that allowed for the heavy and light chain variable regions to be expresed either preceded or followed by the enzyme. Recombinant fusion proteins were expressed in E. coli and purified from periplasmic preparations by immobilized metal affinity chromatography. We observed only low expression levels of soluble 5'-scFv-enzyme-3' fusion protein in the periplasm, which were contaminated by large amounts of the enzyme alone. Enzyme alone was also expressed at about 5-fold higher levels than scFv alone. This has been utilized to increase fusion protein expression by selecting the sequence 5'-enzyme-scFv-3'. Antibody binding was determined by plasmon surface resonance and by flow cytometry with the A33 antigen-expressing colon cancer cell line SW1222. The efficacy of this ADEPT system *in vitro* is demonstrated in a cytotoxicity assay using SW1222 cells cultured in the presence of 5-FC after pre-incubation with the huA33-CD fusion protein.